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(54) Title: PRODUCTS IN THE TREATMENT OF CANCER

(57) Abstract

The present invention encompasses useful products in the treatment of cancer formed by processes utilizing physical and immunologic therapies for the treatment of neoplasms. Products for encouraging host immune responses are formed by conditioning a neoplastic tissue mass with an immunoadjuvant (also called immunomodulator or immunopotentiator) and then physically destroying the conditioned neoplasm in the presence of the immunoadjuvant. Tumor specific antibodies and antigen/antibody complexes are isolated and utilized.

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PRODUCTS IN THE TREATMENT OF CANCER

BACKGROUND OF THE INVENTION

1. Technical Field:

The present invention relates generally to products used in the treatment of cancers.

2. Background:

A neoplasm is an abnormal tissue that grows by cellular proliferation more rapidly than normal. It continues to grow even after the stimulus that initiated its growth dissipates. Neoplasms show a partial or complete lack of structural organization and functional coordination with the normal tissue and usually form a distinct mass which may be either benign or malignant.

Cancer is a general term frequently used to indicate any of the various types of malignant neoplasms, most of which invade surrounding tissues, may metastasize to several sites, and are likely to recur after attempted removal and to cause death of the patient unless adequately treated. Cancer can develop in any tissue of any organ at any age.

Once an unequivocal diagnosis of cancer is made, treatment decisions become paramount. Though no single treatment approach is applicable to all cancers, successful therapy must be focused on the primary tumor and its metastases, whether clinically apparent or microscopic.

CONVENTIONAL TREATMENTS

Historically, local and regional therapy, such as surgery or radiation, have been used in cancer treatment, along with systemic therapy, e.g., drugs.

Surgery is the oldest effective form of cancer therapy. In 1988, about 1,500,000 persons developed cancer; of those, about 515,000 had cancer of either the skin or cervix. About 985,000 had other systemic forms; 64% had operable lesions, with an estimated cure rate of 62%. Cancers that may be positively influenced with surgery alone, if detected in early stages, include those of the cervix, breast, bladder, colon, prostate, larynx, endometrium, ovary, oral cavity, kidney, testis (nonsemino-matous) and lung (non-small cell). It must be noted, however, that the percentage rate of treatment success varies greatly between the cancer sites.

Radiation plays a key role in the remediation of Hodgkin's disease, nodular and diffuse non-Hodgkin's lymphomas, squamous cell carcinoma of the head and neck, mediastinal germ-cell tumors, seminoma, prostate cancer, early stage breast cancer, early stage non-small cell lung cancer, and medulloblastoma. Radiation can be used as palliative therapy in prostate cancer and breast cancer when bone metastases are present, in multiple myeloma, advanced stage lung and esophagopharyngeal cancer, gastric cancer, and sarcomas, and in brain metastases. Cancers that may be curable with radiation alone include Hodgkin's disease, early-stage non-Hodgkin's lymphomas, cancers of the testis (seminomal), prostate, larynx, cervix, and, to a lesser extent, cancers of the nasopharynx, nasal sinuses, breast, esophagus, and lung.

Antineoplastic drugs are those that prevent cell division (mitosis), development, maturation, or spread of neoplastic cells. The ideal antineoplastic drug would destroy cancer cells without adverse effects or toxicities on normal cells, but no such drug exists. Despite the narrow therapeutic index of many drugs, however, treatment and even cure are possible in some patients. Certain stages of choriocarcinoma, Hodgkin's disease, diffuse large cell lymphoma, Burkitt's lymphoma and leukemia have been found to be susceptible to antineoplastics, as have been cancers of the testis (nonseminomatous) and lung (small cell). Common classes of antineoplastic drugs include alkylating agents, antimetabolites, plant alkaloids, antibiotics, nitrosoureas, inorganic ions, enzymes, and hormones.

Despite some success, the above treatments are not effective to the degree desired, and the search has continued for more efficacious therapies.

RECENT ADVANCES

Two of the more recent oncological treatment modalities investigated by the medical community are photodynamic therapy and tumor immunotherapy.

I. PHOTODYNAMIC THERAPY

It has been known for many years that photosensitizing compounds show a photochemical reaction when exposed to light. Photodynamic therapy (PDT) uses such photosensitizing compounds and lasers to produce tumor necrosis. Treatment of solid tumors by PDT usually involves the systemic administration of tumor localizing photosensitizing compounds and their subsequent activation by laser. Upon absorbing light of the appropriate wavelength the sensitizer is converted from a stable atomic structure to

an excited state. Cytotoxicity and eventual tumor destruction are mediated by the interaction between the sensitizer and molecular oxygen within the treated tissue to generate cytotoxic singlet oxygen.

Two good general references pertaining to PDT, biomedical lasers and photosensitizing compounds, including light delivery and dosage parameters, are *Photosensitizing Compounds: Their Chemistry, Biology and Clinical Use,* published in 1989 by John Wiley and Sons Ltd., Chichester, U.K., ISBN 0 471 92308 7, and *Photodynamic Therapy and Biomedical Lasers: Proceedings of the International Conference on Photodynamic Therapy and Medical Laser Applications, Milan, 24-27 June 1992*, published by Elsevier Science Publishers B.V., Amsterdam, The Netherlands, ISBN 0 444 81430 2, both incorporated herein by reference.

United States patents related to PDT include U. S. Patent Nos. 5,095,030 and 5,283,225 to Levy et al.; 5,314,905 to Pandey et al.; 5,214,036 to Allison et al; and 5,258,453 to Kopecek et al., all of which are incorporated herein by reference. The Levy patents disclose the use of photosensitizers affected by a wavelength of between 670-780 nm conjugated to tumor specific antibodies, such as receptor-specific ligands, immunoglobulins or immunospecific portions of immunoglobulins. The Pandey patents are directed to pyropheophorbide compounds for use in standard photodynamic therapy. Pandey also discloses conjugating his compositions with ligands and antibodies. The Allison patent is similar to the Levy patents in that green porphyrins are conjugated to lipocomplexes to increase the specificity of the porphyrin compounds for the targeted tumor cells. The Kopecek patent also discloses compositions for treating cancerous tissues. These compositions consist of two drugs, an anti-cancer drug and a photoactivatable drug, attached to a copolymeric carrier. The compositions enter targeted cells by pinocytosis. The anti-cancer drug acts after the targeted cell has been invaded. After a period of time, a light source is used to activate the photosensitized substituent.

II. TUMOR IMMUNOTHERAPY

The major functions of the immune system are to develop the concept of "self" and eliminate what is "nonself". Although microorganisms are the principal nonself entities encountered every day, the immune system also works to eliminate neoplasms and transplants. See Chapters 18 and 103 of *The Merck Manual of Diagnosis and Therapy*,

Sixteenth Edition, published in 1992 by Merck Research Laboratories of Rahway, N.J., ISBN 0911910-16-6 and 0076-6526; the same being incorporated herein by reference.

There are several distinct types of immunity. Nonspecific, or innate, immunity refers to the inherent resistance manifested by a species that has not been immunized (sensitized or allergized) by previous infection or vaccination. Its major cellular component is the phagocytic system, whose function is to ingest and digest invading microorganisms. Phagocytes include neutrophils and monocytes in the blood and macrophages in the tissues. Complement proteins are the major soluble component of nonspecific immunity. Acute phase reactants and cytokines, such as interferon, are also part of innate immunity.

Specific immunity is an immune status in which there is an altered reactivity directed solely against the antigenic determinants (infectious agent or other) that stimulated it. It is sometimes referred to as acquired immunity. It may be active and specific, as a result of naturally acquired (apparent or inapparent) infection or intentional vaccination; or it may be passive, being acquired from a transfer of antibodies from another person or animal. Specific immunity has the hallmarks of learning, adaptability, and memory. The cellular component is the lymphocyte (e.g., T-cells, B-cells, natural killer (NK) cells), and immunoglobulins are the soluble component.

The action of T-cells and NK-cells in recognizing and destroying parasitized or foreign cells is termed cell-mediated immunity. In contradistinction to cell-mediated immunity, humoral immunity is associated with circulating antibodies produced, after a complex recognition process, by B-cells.

As regards tumor immunology, the importance of lymphoid cells in tumor immunity has been repeatedly shown. A cell-mediated host response to tumors includes the concept of immunologic surveillance, by which cellular mechanisms associated with cell-mediated immunity destroy newly transformed tumor cells after recognizing tumor-associated antigens (antigens associated with tumor cells that are not apparent on normal cells). This is analogous to the process of rejection of transplanted tissues from a nonidentical donor. In humans, the growth of tumor nodules has been inhibited in vivo by mixing suspensions of a patient's peripheral blood lymphocytes and of tumor cells, suggesting a cell-mediated reaction to the tumor. In vitro studies have shown that lymphoid cells from patients with

certain neoplasms show cytotoxicity against corresponding human tumor cells in culture. These cytotoxic cells, which are generally T-cells, have been found with neuroblastoma, malignant melanomas, sarcomas, and carcinomas of the colon, breast, cervix, endometrium, ovary, testis, nasopharynx, and kidney. Macrophages may also be involved in the cell-mediated host's response to tumors when in the presence of tumor-associated antigens, lymphokines or interferon.

Humoral antibodies that react with tumor cells in vitro have been produced in response to a variety of animal tumors induced by chemical carcinogens or viruses. Hydridoma technology in vitro permits the detection and production of monoclonal antitumor antibodies directed against a variety of animal and human neoplasms. Antibody-mediated protection against tumor growth in vivo, however, has been demonstrable only in certain animal leukemias and lymphomas. By contrast, lymphoid cell-mediated protection in vivo occurs in a broad variety of animal tumor systems.

Immunotherapy for cancer is best thought of as part of a broader subject, namely biologic therapy, or the administration of biologic-response modifiers. These agents act through one or more of a variety of mechanisms (1) to stimulate the host's antitumor response by increasing the number of effector cells or producing one or more soluble mediators; (2) to serve as an effector or mediator; (3) to decrease host suppressor mechanisms; (4) to alter tumor cells to increase their immunogenicity or make them more likely to be damaged by immunologic processes; or (5) to improve the host's tolerance to cytotoxics or radiation therapy. Heretofore the focus of cell-mediated tumor immunotherapy has been on reinfusion of the patient's lymphocytes after expansion in vitro by exposure to interleukin-2. One variation includes isolating and expanding populations of lymphocytes that have infiltrated tumors in vivo, so-called tumor-infiltrating lymphocytes. Another is the concurrent use of interferon, which is thought to enhance the expression of histocompatibility antigens and tumor-associated antigens on tumor cells, thereby augmenting the killing of tumor cells by the infused effector cells.

Humoral therapy, on the other hand, has long concentrated on the use of antitumor antibodies as a form of passive immunotherapy, in contrast to active stimulation of the host's own immune system. Another variation is the conjugation of monoclonal antitumor antibodies with toxins, such as ricin or diphtheria, or with radioisotopes, so the antibodies

will deliver these toxic agents specifically to the tumor cells. Active immunization with a host's own tumor cells, after irradiation, neuraminidase treatment, hapten conjugation, or hybridization has also been tried. Clinical improvement has been seen in a minority of patients so treated. Tumor cells from others have been used after their irradiation in conjunction with adjuvants in acute lymphoblastic leukemia and acute myeloblastic leukemia after remission. Prolongation of remissions or improved reinduction rates have been reported in some series, but not in most. Interferons, tumor necrosis factor and lymphotoxins have also been used to affect immunologically mediated mechanisms. A recent approach, using both cellular and humoral mechanisms, is the development of "heterocross-linked antibodies," including one antibody reacting with the tumor cell linked to a second antibody reacting with a cytotoxic effector cell, making the latter more specifically targeted to the tumor. Host immune cell infiltration into a PDT treated murine tumor has been reported.

COMBINED PDT AND IMMUNOTHERAPY

The potential for combining PDT with immunotherapy was explored by Krobelik, Krosl, Dougherty and Chaplin. See *Photodynamic Therapy and Biomedical Lasers*, supra, at pp. 518-520. In their study, they investigated a possibility of amplification of an immune reaction to PDT and its direction towards more pervasive destruction of treated tumors. The tumor, a squamous cell carcinoma SCCVII, was grown on female C3H mice. An immunoactivating agent SPG (a high molecular weight B-glucan that stimulates macrophages and lymphoid cells to become much more responsive to stimuli from cytokines and other immune signals) was administered intramuscularly in 7 daily doses either ending one day before PDT or commencing immediately after PDT. Photofrin based PDT was employed; photofrin having been administered intravenously 24 hours before the light treatment. The SPG immunotherapy was shown to enhance the direct killing effect of the PDT. The indirect killing effect (seen as a decrease in survival of tumor cells left *in situ*) was, however, much more pronounced in tumors of animal *not* receiving SPG. The difference in the effectiveness of SPG immunotherapy when performed before and after PDT suggested that maximal interaction is achieved when immune activation peaks at the time of the light delivery or immediately thereafter. With SPG starting after PDT (and

attaining an optimal immune activation 5-7 days later), it is evidently too late for a beneficial reaction.

In another study the use of PDT to potentiate the effect of bioreactive drugs that are cytotoxic under hypoxic conditions was investigated. See *Photodynamic Therapy and Biomedical Lasers*, supra, at pp. 698-701. It was found that the antitumor activity of such drugs can be enhanced in vivo when they are used in combination with treatments that increase tumor hypoxia.

In PCT International Publication No. WO 96/31237, these inventors disclosed photophysically destroying a tumor while at the same time generating an in situ autologous vaccine to provide a long term humoral immunity against neoplastic cellular multiplication. The photothermal destruction of the tumor also initiates a host immune response. An in situ vaccine is formed when photothermal destruction occurs, as the fragmented tissue and cellular molecules are dispersed within the host in the presence of an immunoadjuvant. The self-immunological defense system is stimulated when this mixture of materials circulates in the host and is detected by the immunological surveillance system. There follows an immediate mobilization of cell mediated immunity, which shifts to a humoral immunity with the production of cytotoxic antibodies over time. Thus, a significant improvement was made in the treatment of cancer whereby the patient is provided with not only immediate tumor destruction but also with the ability to protect the body against a proliferation of residual or metastatic neoplastic cells.

U.S. Patent Application No. 08/720,685 is drawn to novel chitosan-derived biomaterials and their biomedical uses, glycated chitosan being the preferred immunoadjuvant used laser/sensitizer assisted immunotherapy.

OBJECT

It is an object of this invention to further improve the treatment of neoplasms by providing additional procedures that combine direct tumor ablation and immunotherapy in order to induce immediate neoplastic cellular destruction and simultaneously stimulate the self-immunological defense system against residual neoplastic cells.

It is a further object of the invention to provide methods for generating an in situ autologous vaccine and for deriving products useful in cancer related diagnostic and therapeutic procedures.

SUMMARY OF THE INVENTION

Laser/sensitizer assisted immunotherapy encompasses introducing into a neoplasm (1) a chromophore and (2) an immunoadjuvant and then lasing the neoplasm at an irradiance sufficient to induce neoplastic cellular destruction and to stimulate cell-mediated and humoral immune responses.

The present invention is directed to various means of inducing neoplastic cellular destruction while still achieving stimulation of cell-mediated and humoral immune responses and for generating products useful in the treatment of cancer.

In accordance with one aspect of the present invention, an immunoadjuvant is introduced into the targeted tumor to obtain a conditioned tumor and the conditioned tumor is ablated by heat. The hyperthermia may be generated by lasers, ultrasound, microwaves, radiofrequency induction or electric currents.

In another embodiment the conditioned tumor is destroyed by cryotherapy.

Thus, a number of techniques, invasive and non-invasive, are used to induce neoplastic cellular destruction of a tumor conditioned with an immunoadjuvant in order to achieve direct killing of the tumor mass and obtain cell-mediated and humoral immune responses.

The present invention has several advantages over other conventional and unconventional treatment modalities. The most significant advantage is a combined acute and chronic tumor destruction. The acute tumor loss is caused by ablation of the neoplastic tissue, on a large and controlled scale, in the immediate area, reducing the tumor burden and hence the base of multiplication so that the self-defense system can fight a weaker enemy. When this direct destruction occurs, the fragmented tissue and cellular molecules are disbursed within the host in the presence of the immunologically potentiating material, such as chitosan. In effect, an in situ vaccine is formed. This mixture of materials then circulates in the host and is detected by the immunological surveillance system. There follows an immediate mobilization of cell-mediated immunity which encompasses NK-cells and recruited killer T-cells. These cells migrate to the sites of similar antigens or chemicals. In time, the cell-mediated immunity shifts to a humoral immunity with the production of cytotoxic antibodies. These antibodies freely circulate about the body and attach to cells and materials for which they have been encoded. If this attachment occurs

in the presence of complement factors, the result is cellular death. The time frames for these two immunologic modes of action are 0 to 2 weeks for the cell-mediated response, while the humoral arm matures at approximately 30 days and should persist for long periods, up to the life span of the host.

With the present invention collateral damage is reduced to a tolerable level. If a laser is used, the laser power is carefully chosen under a certain damage threshold so that the laser will do little damage to tissue in the path of the leaser beam, such as skin. This characteristic makes a non-invasive treatment possible. Even in the case where diseased tissues are deep inside the body, an endoscope and fiber optics can easily reach the treatment site.

A chromophore of a complementary absorption wavelength makes laser treatment highly selective. Only the chromophore injected area sustains noticeable tissue damage. The concentration of chromophore, the dosage of chromophore and immunoadjuvant, and the timing of administration allow for temporal and spatial control of the induced photothermal effect. The optimal administration can be achieved by considering the physical and chemical characteristics of the chromophore and by considering the tissue responses to the photothermal interaction. Equally important are the natural reactions between the chromophore and its host tissues without any laser stimulation, such as the molecules breaking down over time, as well as the migration of molecules through the circulatory and excretory systems. The preferred chromophore of the present invention, ICG, is non-toxic and can be easily excreted in a short period through the liver and kidney.

In sum, long term survival with total cancer eradication can be achieved by the present invention. It is a combined result of reduced tumor burden due to direct ablation of the tumor mass and an enhanced immune system response due to conditioning the tumor with chitosan or other immunomodulators prior to destroying the tumor.

Still other objects and advantages of the present invention will become readily apparent to those skilled in this art from the following detailed description, wherein there is shown and described only the preferred embodiments of the invention, simply by way of illustration of the best mode contemplated for carrying out the invention. As will be realized, the invention is capable of modifications in various obvious respects, all without

departing from the invention. Accordingly, the description should be regarded as illustrative in nature, and not as restrictive.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a chart related to a study of murine mammary tumors showing tumor burden over time for a particular murine subject whose primary tumor was treated using laser/sensitizer assisted immunotherapy.

FIG. 2 is a chart similar to FIG. 1 for a second treated murine subject.

FIG. 3 is a chart showing tumor burden over time for untreated secondary tumors in the murine subject of FIG. 1.

FIG. 4 is a chart showing tumor burden over time for untreated secondary tumors in the murine subject of FIG. 2.

FIG. 5 is a chart showing tumor burden over time for three other treated murine subjects.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention contemplates combining physical and immunologic therapies for the treatment of neoplasms by conditioning a targeted neoplasm with an immunoadjuvant (also called immuno-modulator or immunopotentiator) and then physically destroying the conditioned neoplasm. A number of techniques or procedures can be used to achieve the physical destruction of the conditioned tumor mass.

A. LASER/SENSITIZER ASSISTED IMMUNOTHERAPY

In this modality the targeted tumor is conditioned with an immunoadjuvant, preferably glycated chitosan, and a chromophore and is then lased, preferably in a non-invasive manner, with a laser having a wavelength of absorption corresponding to that of the chromophore.

The chromophore and immunoadjuvant are preferably combined into a solution for injection into the center of the tumor mass. It should be recognized however that other methods may be sufficient for localizing the chromophore and immunoadjuvant in the tumor site. One such alternative delivery means is conjugation of the chromophore or immunoadjuvant or both to a tissue specific antibody or tissue specific antigen, such that delivery to the tumor site is enhanced. Any one method, or a combination of varying methods, of localizing the chromophore and immunoadjuvant in the tumor site is acceptable

so long as the delivery mechanism insures sufficient concentration of the components in the neoplasm.

CHROMOPHORE

The selection of an appropriate chromophore is largely a matter of coordination with an acceptable laser wavelength of radiation. The wavelength of radiation used must, of course, be complementary to the photoproperties (i.e., absorption peak) of the chromophore. Other chromophore selection criteria include ability to create thermal energy, to evolve singlet oxygen and other active molecules, or to be toxic in their own right such as cis-platinin. In the present invention, the preferred wavelength of radiation is 808 ± 10 nm. The desired chromophores have strong absorption in the red and near-infrared spectral region for which tissue is relatively transparent. Another advantage of this wavelength is that the potential mutagenic effects encountered with UV-excited sensitizers are avoided. Nevertheless, wavelengths of between 150 and 2000 nm may prove effective in individual cases. The preferred chromophore is indocyanine green. Other chromophores may be used, however, their selection being based on desired photophysical and photochemical properties upon which photosensitization efficiency and photocytotoxicity are dependent. Examples of alternative chromophores include, but are not limited to, methylene blue, DHE (polyhaematoporphrin ester/ether), *m*-THPP (tetra(*meta*-hydroxyphenyl)porphyrin), $AlPcS_4$ (aluminium phthalocyanine tetrasulphonate), ZnET2 (zinc aetio-purpurin), and Bchla (bacterio-chlorophyll *a*).

GLYCATED CHITOSAN

The preferred immunomodulator is chitosan. Chitosan is a derivative of chitin, a compound usually isolated from the shells of some crustaceans such as crab, lobster and shrimp. Chitin is a linear homopolymer composed of N-acetylglucosamine units joined by β 1 → 4 glycosidic bonds. Chitin, chitosan (partially deacetylated chitin) and their derivatives are endowed with interesting chemical and biological properties that have led to a varied and expanding number of industrial and medical applications.

The presence of primary and secondary alcohol groups, and of primary amino groups in chitosan, facilitate a number of approaches for chemical modifications designed mainly to achieve their solubilization and to impart special properties for specific applications. Their biodegradability and lack of toxicity renders them "biologically

friendly," since their degradation products can be utilized for the biosynthesis of glycoconjugate components of living tissues. Chitosan and its derivatives have been utilized for bandages and sutures, burn dressings, skin substitutes, bone and dental prostheses, food packaging, drug encapsulation, cosmetics, metal chelation and associated antioxidant effects, waste water treatment, hemostasis, anticoagulants (after sulfation), and dye doping, among other things.

Solubilization of chitin and chitosan can be achieved by partial hydrolysis to oligosaccharides. For chitosan, treatment with a variety of acids, both organic and inorganic, leads to the formation of water soluble chitosonium salts by protonation of the free amino groups. Additional modifications of the amino groups include the introduction of chemical groups such as carboxymethyl, glyceryl, N-hydroxybutyl and others. Glycation, i.e., non-enzymatic glycosylation of the free amino groups of chitosan, followed by stabilization by reduction, offers a novel approach for the preparation of the chitosan gels and solutions utilized in the present invention.

Glycated chitosan, as indicated above, refers to the products resulting from the reaction between the free amino groups of chitosan and the carbonyl groups of reducing monosaccharides and/or oligosaccharides. The products of this reaction (mainly a mixture of Schiff bases, i.e. the carbon atom of the carbonyl group double bonded to the nitrogen atom of the amino group, and Amadori products, i.e. the carbon atom of said carbonyl group bonded to the nitrogen atom of said amino group by a single bond while an adjacent carbon atom is double bonded to an oxygen atom) may be used as such or after stabilization by reduction with hydrides, such as sodium borohydride, or by exposure to hydrogen in the presence of suitable catalysts. The galactose derivative of chitosan is particularly preferred insofar as it has a relatively higher naturally occurring incidence of its straight chain form. The glycated chitosan may be prepared in a powder form, as a viscous suspension, or in other forms.

One protocol for the preparation of glycated chitosan for use in the present invention is as follows: 3 grams of a reducing monosaccharide (e.g., glucose, galactose, ribose), or an equivalent amount of a reducing oligosaccharide, is dissolved in 100 ml of distilled water under gentle magnetic stirring in an Erlenmeyer flask. One gram of chitosan is added. When the suspension is homogeneous, 0.25 ml of toluene is

added, and the flask is sealed with aluminum foil. The magnetic stirring continues for 24 hours at room temperature. After stirring, the suspension is placed in a ventilated fume hood where 1.327 grams of sodium borohydride in 5 ml of 0.1M sodium hydroxide is added to reduce Schiff bases and Amadori products. The solution is then covered loosely with foil, stirred for 10 minutes at room temperature and 50 minutes in an ice bath. After this stirring step, the flask is removed from the ice bath and the solution is acidified to a pH of 5.5 by the dropwise addition of glacial acetic acid (approximately 1.9 ml) under further magnetic stirring to decompose excess borohydride. The solution is then centrifuged for 15 minutes at 15,000 rpm (on a Sorval, rotor SS-34, at 4° C) in five glass (Corex) centrifuge tubes. The supernatant is decanted and the clear gel layer overlaying the pellets is gently scraped with a steel spatula. Using the supernatant from two of the centrifuge tubes, the pellets are resuspended and recentrifuged in two tubes. Again, the supernatant is decanted and the gel is collected as above. The combined pellets are centrifuged a third time after being resuspended in the supernatant of one tube. The gel is again collected after decanting the supernatant. Pooled gel is then dispersed in pooled supernatant to obtain a homogeneous suspension, which is placed in three dialysis bags (Spectrapor, 25 mm flat width, 12,000-14,000 mol. wt. cut-off). The suspensions are dialyzed overnight at 4° C against 3.5 gallons of distilled water. The bags are then placed in fresh distilled water and dialysis is continued for an additional 7 hours. After dialysis, the dialysate is removed from the bags and it is homogenized by 3 bursts (10 seconds each) in a Waring blender at high speed. The resulting viscous solution is stored frozen. Before use in the present invention, the frozen material is thawed in a water bath at 37° C, then mixed in a Waring blender to achieve a homogeneous mix.

Alternatively, 100 ml of 1.0% (by weight of chitosan) galactose derivative of chitosan could be prepared as follows:

1. 250 μ l of glacial acetic acid is added to 100 ml of pure water and the mixture is stirred.

2. 1.00 grams (by dry weight) of chitosan is added. Stirring continues until all of the chitosan dissolves (there will be a few pieces of insoluble non-chitosan impurities). This will take 1-2 hours.

3. 3.00 grams of galactose is added. Stir and let react for 16 hours or more.

4. It should be noted that hydrogen is produced in this step. While vigorously stirring, a 10% sodium borohydride solution (1.0 gram of sodium borohydride dissolved in 0.1N sodium hydroxide brought to 10.0 ml volume) is added while monitoring the pH and the liquid/foam level in the container. When the pH approaches 6.0, 250 μ l of glacial acetic acid is added to lower the pH. The sodium borohydride is added and the pH adjusted until 5.0 ml of the 10% sodium borohydride has been added and the pH is 5.3-5.8. This will require approximately 750 μ l of acetic acid and take about 2 hours.

5. The liquid/foam produced is transferred to centrifuge tubes and is centrifuged for 10 minutes at moderate g (approx. 1500) to break up the foam and separate the particulates. Centrifugation is repeated as necessary (1/2-1 hour).

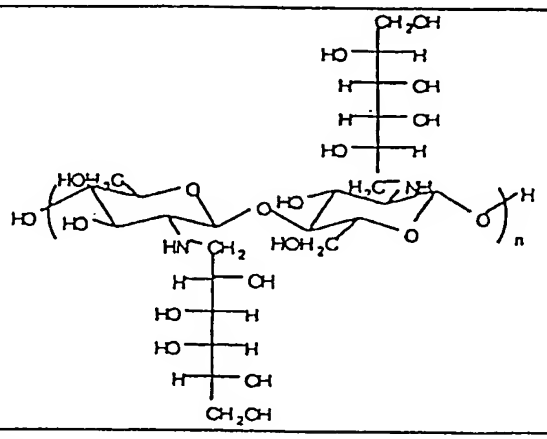
6. The supernatant liquid is then transferred to dialysis tubing (Spectrapor 1) and dialysis is conducted against 4 liters of pure water for 4 hours.

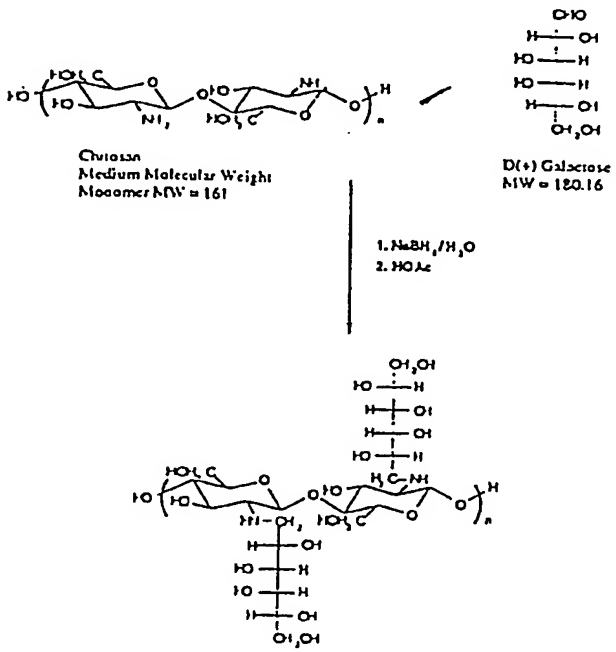
7. To remove excess water acquired during dialysis, the tubing is placed under an air stream (keeping the membrane moist on the inside) for a period of time (2-6 hours) until the weight is reduced the required amount. (20.4mm diameter tubing loses approximately 6g/hour in a fume hood doorway).

8. The dialysis is repeated in 4 liters of water for 6 hours. Then the weight is readjusted in accordance with the previous step.

9. Final dialysis is conducted against 16 liters of pure water for 16 hours, then adjust weight or lyophilize to dryness.

The structure of galacto-chitosan and the preparation of galacto-chitosan from chitosan and D-galactose by reductive ammunition is shown below.

Formula	Biopolymer
Structure	
Molecular Weight (approx.)	1.5 million



USE OF GLYCATED CHITOSAN AS AN IMMUNOADJUVANT

In the preferred embodiment, ICG powder is blended with the glycated chitosan preparation to yield a solution having a concentration of between 0.1% to 2% (.1 to 2 grams/100 ml) of ICG to glycated chitosan solution. The solution is kept warm until use. The effective dosage of the solution ranges from 70 to 2000 μ l. A 100 ml solution of chromophore and glycated chitosan immunoadjuvant also can be prepared from powdered forms of both constituents by added .25 grams of ICG and .5 grams of glycated chitosan to 100 ml of pure water to yield a useful solution having .25% ICG and .5% glycated chitosan by weight. Alternatively, ICG insolution can be mised in appropriate stoichiometric amounts with glycated chitosan solutions to achieve preferred compositions.

As for laser parameters, a solid state diode laser that emits light in a continuous wave through fiber optics of a diameter between 100 and 2000 μ m is preferred, although other lasers may be used, including banks of individual lasers that may or may not all be of the same wavelength. The laser power used can vary between 1 and 60 watts, the preferred power being between 1 and 5 watts. The irradiance duration should last between 1 and 60 minutes, 5 to 15 minutes being favored. The temperature of the lased tumor mass should preferably be raised to about 140° F or 60° C.

In the most preferred embodiment, a solution of ICG and glycated chitosan is prepared as described above at a concentration of 0.25 to 2% of ICG to chitosan. The solution is injected into the center of the neoplasm at a dosage of 70 to 1000 μ l. The neoplasm is then lased using a laser preferably having a power of about 5 watts and a wavelength of radiation capable of readily penetrating normal cellular tissues without significant disruption. The irradiation preferably continues for a duration sufficient to elevate the temperature of the neoplasm to a level that induces neoplastic cellular destruction and liberates tumor antigens which stimulate cell-mediated and humoral immune responses.

OTHER USES FOR GLYCATED CHITOSAN AND DERIVATIVES THEREOF

Other medical and industrial uses for glycated chitosan are anticipated. In addition to its use as an immunoadjuvant or a component thereof in combination with sensitizing dyes and laser/sensitizer assisted immunotherapy, glycated chitosan might be used to facilitate the application of sodium fluoride to the surface of teeth prior to fusion onto the

enamel by laser irradiation. It might also be used to prepare suspensions of hydroxyapatite and other formulations of calcium phosphate utilized for bone and dental prosthesis. Another use would be as a component of skin substitutes, sutures, dressings and bandages for burns, wounds and surgical procedures.

A further use of glycated chitosan, alone or in combination with other drugs, might be as an antiinfection treatment in septicemia, antibiotic resistance, or antibiotic intolerance. It might also be used in cosmetic and pharmaceutical formulations, creams, salves, etc.

A still further use would be as an immunostimulant in the treatment of immuno-compromised patients including but not limited to cancer and acquired immunodeficiency syndrome. This includes, but is not limited to, the use of chitosan derivatives with side chains derived from tumors and microorganisms.

Gel and soluble forms of glycated chitosan will be used individually or in combination, both as such and/or after additional chemical or enzymatic modification. These modifications include, but are not limited to, the generation of reactive groups such as carbonyls and carboxyl groups on the substituents introduced by glycation.

Aldehydes will be generated by oxidation of the carbohydrate side chain (e.g. treatment with periodate or lead tetraaceate) or, for example, the enzymatic oxidation of the primary alcohol group of galactosyl residues with galactosyl oxidase.

Oxidation of the aldehyde groups (e.g. by treatment with hypohalites) will be utilized to obtain the carboxylic acid derivatives. Alternatively, bifunctional compounds containing both free carbonyl and carboxylic groups (e.g. uronic acids) will be utilized during the glycation reaction.

Chitosan deamination with nitrous acid generates reducing aldoses and oligosaccharides suitable for the glycation of chitosan. Deamination of the deacetylated glucosaminyl residues by nitrous acid results in the selective cleavage of their glycosidic bonds with the formation of 2,5-anhydro-D-mannose residues. Depending on the composition of specific areas of the chitosan chain, the anhydro hexose could be released as the monosaccharide, or occupy the reducing end of an oligosaccharide. Release of free N-acetylglucosamine could also occur from some regions of the chitosan chain. Similar treatment of N-deacetylated glycoproteins and glycolipids can be utilized to obtain

oligosaccharides of defined chemical composition and biological activity for special preparations of glycated chitosan. This includes normal as well as pathological glycoconjugates.

The various products obtained by chitosan glycation will be utilized as such or reacted with other natural or synthetic materials, e.g., reaction of aldehyde-containing derivatives of glycated chitosan with substances containing two or more free amino groups, such as on the side chains of amino acids rich in lysine residues as in collagen, on hexosamine residues as in chitosan and deacetylated glycoconjugates, or on natural and synthetic diamines and polyamines. This is expected to generate crosslinking through Schiff base formation and subsequent rearrangements, condensation, dehydration, etc.

Stabilization of modified glycated chitosan materials can be made by chemical reduction or by curing involving rearrangements, condensation or dehydration, either spontaneous or by incubation under various conditions of temperature, humidity and pressure.

The chemistry of Amadori rearrangements, Schiff bases and the Leukart-Wallach reaction is detailed in *The Merck Index*, Ninth Edition (1976) pp. ONR-3, ONR-55 and ONR-80, Library of Congress Card No. 76-27231, the same being incorporated herein by reference. The chemistry of nucleophilic addition reactions as applicable to the present invention is detailed in Chapter 19 of Morrison and Boyd, *Organic Chemistry*, Second Edition (eighth printing 1970), Library of Congress Card No. 66-25695, the same being incorporated herein by reference.

EXPERIMENTAL STUDIES

Further description of laser/sensitizer assisted immunotherapy utilizing a chitosan-derived immunoadjuvant, including components, parameters and procedures, is contained in the following summary of a study of murine mammary tumors.

Materials and Methods:

1. The Laser

A diode laser was used in this study. The Industrial Semiconductor Laser ISL50F (McDonnell Douglas Aerospace, St. Louis, MO), employs a diode array that is electrically powered by a diode driver. The laser emits radiation at wavelength of 808 $\pm$ 10 nm, either

in pulsed or in continuous mode. Its maximum near infrared power in the available configuration output is 35 watts. The laser is operated with standard electric power (110/120 VAC). A microprocessor monitors and adjusts the laser operating parameters. Laser energy can be delivered through optical fibers of various sizes. A red laser diode emits 670 nm light at 0.9 mW as the aiming beam. The continuous wave mode was employed in this experiment.

In the experiment, different power and duration of laser irradiation, ranging from 3 minutes at 5 watts to 5 minutes at 15 watts, were used. The output power was measured before, during and after the procedure using a Joule/Watt meter (Ophir Optics, Israel). Two types of fiber optics were used: 600 μ m and 1200 μ m in diameter.

2. The Preparation of Animals

Wistar Furth female rats, age 6 to 7 weeks and weighing 100 to 125 grams, were chosen for the study. The model is a metastatic transplantable rat mammary tumor. The tumor strain was the DMBA-4. Tumor cells (about 25,000 cells) were transplanted to the rats by injection into the superficial inguinal area between the skin and the muscle layer. Certain selected rats were injected with tumor cells in both left and right inguinal areas for simultaneous tumor growth.

The rats were fed a special food, a high saturated fat diet, to facilitate the growth of the tumor. The tumor usually grew to about 1 to 4 cm^3 within 10 to 14 days of tumor transplantation. In most cases the rat tumors were treated before they grew beyond 5 cm^3.

Before laser treatment, anesthesia was applied (100 μ l xylazine and ketamine solution IM) and the hair overlaying the tumor was clipped and shaved. After the treatment, the rats were maintained in separate cages and still fed with the same high fat diet. The rats were observed daily and the morphological measurements of tumors -- both laser treated and untreated controls -- were made twice a week.

3. Sensitizer Administration

The sensitizer indocyanine green (ICG) (Sigma Chemical Co., St. Louis) was used in two forms: ICG in water and ICG in glycated chitosan, the concentration being 0.5% and 1% (g/100 ml) ICG to water or chitosan. In the case of chitosan solution, an ICG powder was mixed with glycated chitosan made as hereinabove described, after the gel was brought to 37° C from frozen state (-4° C), in a glass grinder to obtain a uniform solution.

The sensitizer solution then was injected into the center of the targeted tumor, either 24 hours prior to laser treatment or just 10 minutes before the procedure. The dosage varied between 70 μ l to 400 μ l to one tumor.

For the rats with transplanted primary tumors in both inguinal areas, only one tumor received the ICG-Chitosan injection. However both tumors were treated using the same laser parameters.

4. Laser Treatment

The laser energy was delivered to the treatment sites through fibers having diameters of 600 or 1200 μ m. The tip of the fiber was maintained 4 mm from the skin. The fiber tip was moved evenly and slowly through all sides of the tumor to ensure a uniform energy distribution. A thin water film (20° C) was constantly applied on the surface of treatment sites to prevent unnecessary damage of skin due to the surface heat build up.

For the rats with two simultaneous primary tumors, one tumor was lased with the aid of ICG-Chitosan and the other was treated by the laser only. Fifty-six rats were treated using various laser parameters in conjunction with ICG-Chitosan solutions. Sixteen rats were injected with 100-200 μ l of a 1% ICG-H_2O solution and lased with various irradiances from 3-5 minutes and from 3-10 watts.

Results:

1. The Survival Rates

In total, one hundred five (105) rats were used in the study up to April 1, 1995. All the rats were injected with the metastatic transplantable rat mammary tumor cells, either in one inguinal region or both. The rats were divided into three groups: (1) control, (2) laser treated with ICG-H_2O solution, and (3) laser treated with ICG-Chitosan solution. The survival rates of the various grouped subjects are summarized on Table I.

Table I

The Survival Rate of Rats with Tumor Transplant

Group	Survival Days	Number of Rats
Control	32.4±3.6	13
Laser Treated with ICG only	29.0±3.5	7
Laser Treated with ICG-Chitosan	42.7±28.9	55
ICG-Chitosan Treated (w/o long survival rats)	32.9±5.7	49
ICG-Chitosan Treated (long survival rats only)*	114.3±39.8	6
ICG-Chitosan Treated 5w @ 3 to 6 mins (w/o long survival rats)**	33.6±6.0	36
ICG-Chitosan Treated 5w @ 3 to 6 mins (with long survival rats)	45.4±32.4	42

* Out of the six long term survival rats, three are still alive. The data collected in this table were up to January 16, 1995.

** The six long survival rats were treated using this laser power and the duration range (see Table II for more detail).

The average survival time for the 33 control rats was 31.5 days after the tumor transplant; the average was 29.0 days for laser treated rats injected with aqueous ICG only; the overall average survival time was 45.6 days for the ICG-Chitosan injected laser treated rats. Among the 56 rats in the last group, six rats achieved long term survival (at least twice that of the control rats), without which the group survival rate would be 32.8 days. On the other hand, the six rats gave rise to an average 152.0 days of survival. It is worthwhile to note that three rats, Srat3, Srat4 and Srat6, were still alive when the report was written after they have survived 220, 192 and 147 days, respectively, up to April 1, 1995.

2. The Tumor Responses to Laser Treatment

Table I shows that tumor rats in both group 2 and group 3 responded positively to laser energy. Almost all the tumors had temperature elevation immediately after the laser treatment. The ICG-H_2O or ICG-Chitosan solution injected tumors usually raised temperature by 40° F, while the ICG free tumor still raised temperature but at a lower level, usually 20° to 30° F above the body temperature depending on the laser power and duration.

Internal explosions often occurred during the procedure due to the sudden temperature increase. It was more evident under high laser power, above 10 watts for example. High power produced skin damage in most cases, even with constant application of water droplets on the treatment surface.

The tumor cell destruction under high laser power (10-15W) was rather superficial and deeper tumor cells often survived the laser assault. Better results were obtained when lower laser powers were applied. All the six long term survival rats resulted from the 5 watt treatment (4 rats with 3 minute exposure times and 2 rats with 5 minutes). See Table II.

Table II

Parameters for Laser-ICG-Chitosan Treated Long Term Survival Rats

Animal	Tumor Transplant		ICG-Chitosan Administration			Laser Treatment*			Survival
Rat Number	Date	Locations	Time Prior to Treatment	Dosage	Location	Date	Power (Watts)	Duration (minutes)	Post Tumor Transplant (Days)
Srat1 Rat #5/3-29-94	3-18-94	Both Legs	24 hours	70 μl @ 1%	Left Leg	3-29-94	5.00	3	156
Srat2 Rat #1/6-3-94	5-23-94	Both Legs	24 hours	70 μl @ 1%	Left Leg	6-3-94	5.00	3	125
Srat3 Rat #5/9-1-94	8-19-94	Left Leg	24 hours	150 μl @ 0.5%	Left Leg	9-1-94	5.06	5	150** as of 1/16/94
Srat4 Rat #2/9-27-94	9-16-94	Both Legs	24 hours	100 μl @ 1%	Right Leg	9-27-94	5.20	5	120** as of 1/16/94
Srat5 Rat #3/11-8-94	10-31-94	Left Leg	10 minutes	150 μl @ 0.5%	Left Leg	11-8-94	4.95	3	65
Srat6 Rat #4/11-11-94	10-31-94	Left Leg	10 minutes	100 μl @ 0.5%	Left Leg	11-11-94	5.12	3	80** as of 1/16/94

* Totally 42 rats were treated using parameters in this range, resulting in a 14% survival rate.

** Rat is still alive.

In total, 43 rats were treated with laser power of 5 watts and an irradiation duration of 3 to 6 minutes; this gives rise to a 14% long term survival rate in this group. Under these parameters, without the six long survival rats, the average survival reached 32.8 ± 6.3 days.

Immediately after laser treatment, all the tumors showed a slower growth within the first few days, then returned to a normal growth rate. Often the tumors would be partially bitten or chewed, but that would not stop or slow the tumor growth. Most rats died around 30 to 35 days except for the rats listed in Table II. About half the treated rats later developed secondary tumors, most as "hand bags" (metastatic to lymph nodes in the axillary region); the local expansions around the primary tumors were also in lymph nodes. In either case, the secondary tumors continued to grow until death occurred, except for the rats in Table II.

3. Tumor Development of the Long Term Survival Rats

All six rats in Table II were treated using 5 watts power with either three minutes duration (four rats) or five minutes (two rats). More importantly, all of them were injected with ICG-Chitosan gel solution. For rats with two transplanted primary tumors, only one of the two tumors received the ICG-Chitosan solution and the other was lased without any ICG sensitizer. For rats with only one primary tumor, the ICG-Chitosan was used. The ICG-Chitosan solution was 0.5% to 1% and the dosage varied from 70 μ l to 150 μ l per tumor. The ICG-Chitosan was usually injected directly into the tumor either 24 hours or 10 minutes before the laser treatment.

Like all the other rats, the tumors continued to grow after treatment, and most of the tumors metastasized along the milk line to the axillary nodes and the opposite inguinal nodes. However, the development of tumors took a dramatic turn at a later stage. Shown in FIGS. 1 and 2 are the growth charts of the primary tumors of Srat1 and Srat2, treated by laser; the left inguinal tumor was injected with ICG-Chitosan and the right inguinal tumors were not. The growth reached a peak around 50 days after the tumor transplant, and then started to regress. The tumors continued to shrink and reached their minimum size around 90 days. Afterwards the tumors started growing again, with Srat1 being more aggressive than Srat2. Note the bigger burden on the right inguinal in both cases (no ICG-Chitosan injection).

The secondary tumors of Srat1 and Srat2 appeared around day 20 after tumor transplant, and went through the same pattern as the primaries -- growth/shrinkage/ growth, as shown in FIGS. 3 and 4. In the case of Srat2, the recurrence of secondary tumors was almost negligible.

The tumor growth of three other rats is shown in FIG 5. Srat3 and Srat6 started with only one primary tumor and Srat4 with two. FIG. 5 shows a much earlier response: tumor reduction started around 20 to 25 days. Furthermore, there are so far no secondary tumors, and the primary tumors have become only a small hard core of fibrous tissue; just a remnant of the tumor.

4. Improvement of the Experiment

Early experiments had been mainly focused on establishing workable conditions, including laser parameters, as well as the concentration and dosage of ICG-Chitosan. No long term survival was observed in the first few groups. Suggested by in vitro and in vivo results, work had been proceeding in the laser power range of 3 to 5 watts. ICG-Chitosan solution between 0.5% to 1% seem to be effective. A steady long term survival rate of 10% has been achieved, and even a 20% rate in one of the recent rat groups.

Discussion:

Clearly, the photothermal effect of the 808 nm diode laser on organized tissue can be greatly enhanced when the chromophore ICG with an absorption peak around 800 nm is used. The 808 nm laser energy can penetrate readily through the normal tissue leaving the cellular structure largely intact within regulated power ranges.

It is the ICG molecule, when injected to the target tissue, that absorbs strongly the 808 nm radiation and reaches an excited state. When the molecule returns to ground state, the stored energy is released in the form of heat which can be absorbed by surrounding tissue to elevate temperature. (The excited ICG molecule may also cause other biochemical reactions which may be the key in our induced immunological responses.) When a sufficient amount of ICG molecules are excited within a certain time (normally shorter than the tissue thermal relaxation time), the released heat can be absorbed by tissue cells faster than it can be dispersed. If the exposure to laser is long enough, the accumulated heat energy can raise tissue temperature to a level at

which photothermal destruction of organized tissue can occur. This destruction can be achieved with certain selected laser powers and irradiation durations. It appears that a laser power around 3 to 5 watts is sufficient to cause fatal injury to tumor cells.

Higher powers, in conjunction with ICG, can cause quick and more drastic thermal injury to the tissue, but some undesirable results may arise. The high power irradiation gives rise to a much faster temperature build-up, often leading to internal explosions and quick tissue carbonization, particularly on the treatment surface when oxygen is abundant in the air. A surface cooling procedure, either by water film or by helium gas may not be able to slow down the carbonization, which changes the surface tissue properties from almost transparent to highly absorbent to the 808 nm wavelength. The 808 nm radiation would be in turn absorbed further by the carbonized tissue. This would impede the penetration of the laser energy, resulting in a superficial and limited spatial thermal destruction of intended malignant tissue.

The thermal impact alone may slow down the short term tumor growth, but may not alter the predestined fate for hosts who have acquired tumors. As shown in Table I, the second group, treated by laser with the aid of ICG-H_2O solution, did not show any improvement on the survival rate. Of course, the destruction of tumor cells due to photothermal interaction was a predominant effect. However, due to the aggressive nature of the tumors, total eradication was rarely achieved by the thermal destruction alone; just as in the cases of surgical removal and radiation therapy.

CONCLUSION

It is thus apparent that other mechanisms must be utilized in order to deal with the root cause of the malignant cell multiplication. The ideal mechanism, of course, is the self-immunological defense system, which can prevent growth of abnormal cells. It is when the natural immune functions are debilitated, or not adequate in response to foreign element invasion, that cancers occur. If natural immune defense failed to stop the uncontrolled growth of cells, endogenous or exogenous, stimulated immune responses are needed. As previously stated, it has long been known that certain chemicals may enhance the natural defense mechanism, but often the enhancement is one or several steps behind the tumor growth. The model described herein was directed to a combined PDT and immunotherapeutic treatment.

Other modalities, such as chemotherapy and radiation therapy, often kill cells indiscriminately so that the collateral damage can be just as fatal. And though PDT relies on heat and/or toxic singlet oxygen generated by treatment of laser and certain photosensitizers to kill tumor cells, it still is not a means to affect the host's self-immunological defense system, other than just incidentally. By using a combination of laser, chromophore, and immunomodulator, a novel cancer treatment is provided.

The effect of this invention in stimulating cell-mediated and humoral immune responses in the host is shown FIGS. 1-5. The growth charts (FIGS. 1 and 2) show the change of the tumor burden. Fifty days after tumor transplantation (40 days after laser-ICG-Chitosan treatment), primary tumors reached their maximum sizes. The reduction of tumor growth and size afterwards can only be explained by the generated immunological response, since only the self-defense mechanism, when fully developed, would slow down and stop the tumor growth, and the disfunctioning tumor cells would be engulfed by macrophages. Without this exotic feature, none of the rats could have survived over 35 or 40 days, as demonstrated by the first two groups of experimental rats in Table I.

The ICG-Chitosan injected tumor (left inguinal in most cases of the six rats in Table II) had a slower growth rate, a sign of a more direct impact of the immunoadjuvant, even though both tumors were treated with same laser power and duration. The ICG-Chitosan injected tumor in general responded more to the laser treatment. The thermal interaction alone, in conjunction with ICG, had effectively reduced the tumor burden on a large scale. The chitosan apparently added the immunological stimulation. The combination of the thermal and immunological effects appear to be the explanation as to why the left inguinal tumors had less growth than those of the right as shown in FIGS. 1 and 2.

The generation and acceleration of the immunological defense system response is further supported by the evolution of the secondary tumors of the long surviving rats. The metastasis usually occurred to half the rats around 15 to 20 days after the transplantation of primary tumors. The secondary tumors appeared in most cases along the milk lines and continued to grow until death. However, the metastatic tumors of

Srat1 and Srat2 in FIGS. 3 and 4 showed exactly the same trend as in the primaries (FIGS. 1 and 2), with neither ICG-Chitosan injection nor laser treatment. FIG. 5 depicts the growth of primary tumors of three rats (Srat3, Srat4 and Srat6), all of them following the same development -- growth/treatment/growth/ reduction. Furthermore, these three rats developed their full responses earlier; the tumor growth was stopped around 20 to 25 days after tumor transplantation and secondary tumors never appeared. This early establishment of the induced immunological defense mechanism may explain why these rats are still alive and have no signs of tumor recurrence.

In conclusion, long term survival with total cancer eradication can be achieved by laser-chromophore-adjuvant induced immunological responses. It is a combined result of reduced tumor burden due to photothermal interactions and an enhanced immune system response due to the addition of chitosan or other immunomodulators. The experimental results have been improving constantly. The first few groups yielded no long term survivors whereas a steady 10% long term survival rate is now achieved. Within the current adapted laser power (5 watts) and the irradiation duration (3 to 6 minutes), the long term survival rate reaches up to 14%.

B. ALTERNATIVE PHYSICAL THERAPIES

Nonthermal cytotoxic phototherapy, as described above, usually involves the systemic administration of tumor localizing photosensitizing compounds and their subsequent activation by laser. Upon absorbing light of the appropriate wavelength the sensitizer is converted from a stable atomic structure to an excited state. Cytotoxicity and eventual tumor destruction are mediated by the interaction between the sensitizer and molecular oxygen within the treated tissue to generate cytotoxic singlet oxygen.

Neoplastic cellular destruction, however, can also be achieved by subjecting the neoplasm to heat energy. Possible mechanisms for thermal cell death include direct damage to DNA, cell membrane damage, the heat stimulated production of special proteins, and disruption of the microvasculature of the tumor. See S.G. Brown, MD, *Phototherapy of Tumors*, World Journal of Surgery, Vol. 7, pp. 700-709 (1983). The thermal effects of photocoagulation and cellular vaporization produced by hyperthermia are believed to be sufficient to induce neoplastic cellular destruction and to generate fragmented neoplastic tissue and cellular molecules such that, when in the presence of

an immunoadjuvant, would form an in situ vaccine as contemplated by the present invention. Means of inducing cellular hyperthermia include the use of lasers, ultrasound, microwaves, radio frequency induction or electric currents.

One laser treatment modality is that of stereotaxic interstitial laser therapy (ILT). Interstitial laser photocoagulation or vaporization depends on the bare end of a laser fiber being inserted into a tumor mass. The laser light is then absorbed as heat with the production of a region of necrosis around the fiber tip. This is, thus, an invasive technique. The use of ILT in the treatment of breast cancer was described by Dowlatshahi, et al. in *Stereotaxic Interstitial Laser Therapy of Early Stage Breast Cancer,* The Breast Journal, Vol. 2, No. 5, pp. 305-311 (1996) and by Harries, et al. in *Interstitial Photocoagulation as a Treatment for Breast Cancer,* British Journal of Surgery, Vol. 81, pp. 1617-1619 (1994), both publications being incorporated herein by reference.

Typically, ILT implores the use of a laser, such as an 805 nm diode laser and a light transmissive fiber, such as a 400 μm quartz fiber. Under general or local anesthesia a needle is inserted into the tumor and the tip of the needle is positioned within the center of the tumor using ultrasonography to monitor the needle position. The laser fiber is advanced through the canula of the needle and the needle is then withdrawn slightly so that the tip of the fiber lays within the tumor. The tumor is then treated with laser light at a power and for an exposure time sufficient to cause neoplastic cellular destruction. Thermocouples may be used to display the intratumor temperature in real time. Apparatae and methods for conducting ILT are described in U.S. Patent Nos. 5,169,396; 5,222,953 and 5,569,240, which patents are incorporated herein by reference.

Tumor hyperthermia can also be achieved by treating a tumor with high intensity ultrasound. Ultrasound is a penetrating, directional and even focusable radiation. High energetic focused ultrasound for therapeutic use involves using flexible ultrasound equipment for tissue destruction by generating small hot spots with one or more focused transducers. This could be accomplished, depending upon tumor location, as an invasive or non-invasive technique. The biological effect of ultrasound involves both heat and cavitation. A coupling medium may be used to transmit the

ultrasonic beam(s) to tissue. Both invasive and non-invasive techniques of applying heat energy have been developed for microwave and radio frequency heating. With radio frequencies the non-invasive applicators are either capacitive plates or inductive coils. With microwaves radiative apertures are used. Invasive applicators include the use of a radiating monopoles for microwaves and RF needle electrodes or implanted ferromagnetic seeds (selectively heated by external inductive coils) for radio frequencies. The penetration and severity of damage can be controlled by the frequency and total energy of the applied radiation. The volume of tumor that can be heated is comparable to that heated using a Nd:YAG laser with the transmission fiber inserted into the tumor (radius of 1-2 cm from the treatment point). The radiating monopole is a miniature coaxial transmission line which can be made flexible and implanted surgically or inserted via body orifices. See S.G. Brown, *Phototherapy of Tumors, supra.*

One other potential way of causing neoplastic cellular destruction by hyperthermia would be to circulate heated water about the tumor in order to raise the temperature of the tumor. This could be done on peripheral tumors that are approachable with a device from the outside, but this approach might not be too effective at high temperatures.

Cryotherapy may also be used to achieve neoplastic cellular destruction. Cryotherapy is the use of extreme cold to destroy cancer cells. Cryotherapy was first used in treating external tumors, such as those on the skin, by contacting the skin with liquid nitrogen. For internal tumors a cryoprobe can be used to circulate liquid nitrogen into contact with the tumor. Ultrasonography may be used to guide the cryoprobe and monitor the freezing of the tumor mass. Cryotherapy has heretofore been used in the treatment of, among other things, prostate cancer and liver metastases. It is sometimes used in combination with other cancer treatments such as radiation, surgery, and hormone therapy. As with other mechanisms of tumor destruction, the focus is on destroying the tumor without damaging nearby healthy tissue.

It is thought that several processes contribute together to achieve cryosurgical cellular destruction. The creation of intracellular ice by rapid temperature loss is fatal to the cells. Moreover, as ice forms around a cell the free water inside the cell is

drawn off shrinking the cell and collapsing the walls or membranes inside the cell, releasing proteins or chemicals which can be toxic. In addition, as ice which surrounds shrunken cells begins to thaw, large amounts of free water produced by the thawing ice will rush back inside the cells, causing them to burst. Thus, it is believed by some that the physical features of cryosurgery which are most important in producing extensive cell destruction include rapid freezing to very low (-195°C) temperatures, and a slow thawing.

Cryotherapy has heretofore been used in treating prostate cancer, skin disorders and retinopathy in premature infants. As with hyperthermia, thermocouples can be used to monitor temperature to confirm that all areas of the tumor are properly frozen.

C. SPECIAL PRODUCTS IN THE TREATMENT OF CANCER

Certain special products produced by the interaction of tumor antigens and the adjuvant with the host may be derived from the described therapies. It is anticipated that the present invention will be particularly useful in the treatment of breast tumors, prostate cancers, lung cancers, melanoma, AIDS (treating nodes to immunize against the virus) and squamous cell carcinomas in humans. It is further anticipated that with respect to human treatments, certain in vitro procedures will be useful. For example, a neoplasm may be removed from a tumor-bearing host, treated in accordance with the above described procedures in the lab, and products returned to the host for subsequent administration.

One product would be a mixture of fragmented neoplastic tissue and cellular molecules and an immunoadjuvant, the fragmented neoplastic tissue and cellular molecules having antigens exposed thereon. In essence, a tumor would be removed from the host, prepared or conditioned (for example with a chromophore and immunoadjuvant) for physical destruction, then physcially fragmented to expose tumor antigens (such as by laser) in the presence of the immunopotentiator, as described in detail above. The resulting product would then be administered back to the host to encourage an immune response.

Another product would be specific isolated antibodies directed against certain antigenic domains found on the cell surface. When the antibodies and antigens meet a complex is formed that can be separated and identified. The separated products then

can be used as probes to determine if the antigens are common to all human tumors of the same type. The tumor specific antibodies may also be isolated, evaluated and utilized for the ability to fix complement and to direct complement-mediated cytotoxicity and to mediate antibody-dependent cellular toxicity, as further described below.

Likewise, free antibodies can be used to collect circulating free antigens for identification and possible synthesis to form the basis of a screening test for very early breast cancer.

It is also possible for these antibodies to be used to locate and treat recurrent or metastatic tumors in areas not approachable by the original technique. This is accomplished by tagging the antibody with chemicals that aid in localization or drugs that are toxic to cells.

Furthermore, the serum from cured hosts could be injected into untreated tumor-bearing hosts in order to induce adoptive immunity. The serum from cured hosts could also be injected to healthy hosts in an attempt to prevent tumor development.

Thus, a large variety of products could be generated from the reaction of the host to the products of the described treatment. Likewise these products may be keys to other methods to attack tumor cells, both directly and indirectly.

A recent experiment utilizing female Wistar Furth rats performed in accordance with the above described modality resulted in a 38% long term survival — 120 days after tumor implantation, a 300% increase in survival length compared to untreated control tumor-bearing rats. Primary tumors of successfully treated rats usually continued to grow, and at a certain point after treatment (about 4 to 6 weeks) the tumor burden began to decrease. The tumor totally disappeared in 9 to 12 weeks. All the tumor-bearing rats developed metastases in the remote inguinal and axillary areas two weeks after inoculation of the primary tumor. The untreated metasteses of the cured rats also regressed with the same pattern.

The preliminary studies showed that the treatment of this metastatic tumor model was effective only when all three components, namely, the laser, ICG and glycated chitosan gel, were applied together. Injection of ICG alone, laser irradiation alone, or use of laser-ICG combination did not increase the survivability of the tumor-

bearing rats. Use of glycated chitosan gel alone or ICG-GCG or laser-GCG combinations achieved only limited improvement, far less than that by the preferred modality.

To determine the long-term effect of the treatment, the successfully treated rats were rechallanged by increased tumor dosage of 10^6 viable cells (10 times the original tumor dosage in inoculation). None of the rats developed tumors. In contrast, all age-matched control tumor-bearing rats died within 35 days. The hypothesized mechanism of action in the successfully treated rats is a tandem effect resulting from the photothermal and photoimmunological interactions. The photothermal reaction reduces the tumor burden and, at the same time, exposes the tumor antigens. Then the immune system, enhanced by the immunoadjuvant, is able to recognize the exposed antigens and mount a systemic attack on the cells of remaining tumors and the metastases. Without laser-ICG photothermal destruction, the immune system may not be able to recognize the specific antigens of a well-masked tumor cell; without the immunoadjuvant stimulation, the native immune system may not react fast enough or summon enough strength to control the tumor, even a reduced or disrupted tumor. Moreover, this immunity is induced in the individual host through local tumor treatment. The treatment could, in effect, produce an in situ autovaccine, without traditionally required antigen cross-reactivity.

Sera obtained from successfully treated tumor-bearing rats were analyzed for antibodies that bound to tumor cells. Two histochemical assays were performed. The first was a living cell fluorescent assay which allows the detection of antibodies that bind to the plasma membrane of isolated live tumor cells. The second assay used preserved tumor tissue and the peroxidase reaction product to determine antibody binding to the plasma membrane and other cellular antigens. Both assays showed strong antibody binding compared with that from sera from untreated control tumor-bearing rats.

In the living tumor cell fluorescence assay, sera from control tumor-bearing rats and from rechallenged cured rats were diluted 1:1000 in phosphate buffered saline. Freshly collected tumor tissue was dispersed to a single cell suspension by grinding in a loose-fitting ground glass homogenizer. The single cells were incubated with the

diluted serum for one hour, then raised three times in phosphate buffered saline followed each time by low speed centrifugation. The cells were then incubated in a secondary fluorescein labeled goat anti-rat antiserum for one hour, then rinsed in phosphate buffered saline three times. The cells were then mounted in aqueous mounting medium and viewed immediately with a fluorescence microscope. There was little, if any, fluorescence in the cell stained by serum from untreated tumor-bearing rats. In contrast, a tumor cell stained with serum from the cured tumor-bearing rat shows a high fluorescence and uniform staining over the plasma membrane.

In the tumor tissue immunoperoxidase assay, tumor tissue was fixed in 2% paraformaldehyde, then dehydrated and embedded in paraffin. Sections were cut and mounted on glass slides, then rehydrated. The sections were incubated for one hour with the diluted serum (1:1000) from untreated tumor-bearing rats (32 days after tumor transplantation) and from successfully treated tumor-bearing rats (32 days after tumor rechallenge), respectively. Then, the sections were rinsed three times in phosphate buffered saline. After the final wash, the sections were labelled with peroxidase with the Vector ABC kit and viewed with an optical microscope. Photomicrographs of tumor sections from the untreated tumor-bearing rats lacked the brown reaction product that indicates peroxidase activity. In contrast, intense staining at the plasma membrane (and a lack of staining within the cells) was shown in photomicrographs of tumor sections from the successfully treated tumor-bearing rats.

Study of Cell-Mediated Immune Responses:

A standard chromium release assay will be used to detect laser-ICG-GCG induced tumor-specific cytotoxic T cells, with viable tumor cells being the targets and rat peripheral blood or lymph node lymphocytes being the effectors. Viable tumor cells will be collected from untreated tumor-bearing hosts and isolated by Ficoll Hypaque density gradient centrifugation. The cells will be labeled with $Na_2[^{51}Cr]O_4$ (50 μCi/10^6 cells) by incubating for 45 minutes in a 37°C water bath. The labeled cells will be separated from unicorporated ^{51}Cr by centrifugation through fetal calf serum.

The effector cells will be peripheral blood mononuclear cells from healthy hosts, untreated tumor-bearing hosts, and laser-ICG-GCG treated tumor-bearing hosts. Blood

will be collected at designated times after tumor transplantation and treatment. Peripheral blood mononuclear cells will be isolated by Ficoll Hypaque density gradient centrifugation, washed, and resuspended in complete medium at desired concentrations. In other experiments, cells from lymph nodes in the proximity of the tumor or from tumor infiltrates will be used as effectors.

^{51}Cr-labeled tumor cells (4000 cells in 25 μl) and various concentrations of effector cells (in 200 μl) to yield effector:target ratios of 10:1 to 100:1 will be added to U-bottomed microtiter plates in triplicate. After low speed centrifugation, the cells will be placed in an incubator containing 5% CO_2 in air for 4 hours at 37°C. The supernatant of 150 μl containing ^{51}Cr released by tumor cells killed by cytotoxic T cells will be removed from each well and counted in a gamma counter. The controls will be 25 μl of labeled tumor cells added to 200 μl of complete medium to yield "spontaneous" release and 25 μl of labeled tumor cells added to 200 μl of 1% Triton X-100 + 1M KOH to yield "maximum" release. The data will be calculated as follows:

$$\%^{51}\text{Cr release} = \text{ER-SR/MR-SR} \times 100\%,$$

where MR = maximum release, ER = effector-induced release, and SR = spontaneous release. The indication of the cytotoxity of T cells from laser-ICG-GCT treated hosts would be a significant increase of ^{51}Cr release, comparing with that of T cells from heathy hosts and from untreated tumor-bearing hosts.

Study of Humoral Immune Responses:

a. Detection of antibodies against tumor cells Plasma from treated and untreated tumor-bearing hosts may be examined for the presence of tumor-specific antibodies by indirect immunofluorescence. Diluted plasma (50μl) can be incubated with 10^6 viable tumor cells (isolated as described above) for 30 minutes at 4°C. The cells will then be washed with 1 ml of PBS = 0.1% sodium azide and then incubated with 50 μl of FITC-goat anti-rat Ig for 30 minutes at 4°C. After washing, the amount of antibody bound to the cells will be determined with a Becton Dickinson FACScan Fluorescence activated cell sorter. Plasma from untreated tumor-bearing hosts will serve as a negative control. If positive fluorescence is observed, the specificity of the reaction will be determined by staining a panel of host tumors to which the treated hosts were never exposed.

b. Complement-mediated tumor destruction The antibodies will be evaluated for the ability to fix complement and to direct complement-mediated cytotoxicity. The technique used for this assay is a modification of the same ^{51}Cr release assay as described above. Briefly, ^{51}Cr-labeled tumor cells will be incubated with diluted serum from treated and untreated hosts for 30 minutes in ice. A source of complement (such as baby rabbit serum) will then be added and the cells will be incubated at 37°C for 1 hour. The cells will then be centrifuged and the amount of radioactivity released will be measured with a gamma counter.

c. Antibody-dependent cellular cytotoxity The tumor specific antibodies will also be evaluated for the ability to mediate antibody-dependent cellular cytotoxicity (ADCC). This assay is also a modification of the same ^{51}Cr release assay as described above. Labeled tumor cells will be added to U-bottomed microtiter wells along with dilutions of serum from control and treated tumor-bearing hosts as a source of antibodies and host spleen or lymph node cells as a source of effector cells. After incubation at 37°C for four hours the amount of cell lysis will be evaluated by measuring the quantity of radioactivity released into the supernatant with a gamma counter.

Thus, it can be seen that a variety of products for the treatment of cancer are made available in connection with the present invention.

While the invention has been described with a certain degree of particularity, it is manifest that many changes may be made in the method hereinabove described without departing from the spirit and scope of this disclosure. It is understood that the invention is not limited to the embodiments set forth herein for purposes of exemplification, but is to be limited only by the scope of the attached claim or claims, including the full range of equivalency to which each element thereof is entitled.

WHAT IS CLAIMED IS:

1. An in vitro method for preparing a cancer treatment product, comprising the steps of:

(a) introducing an immunoadjuvant into a neoplastic tissue mass to obtain a conditioned neoplasm; and

(b) performing a physical therapy upon said conditioned neoplastic tissue mass to induce neoplastic cellular destruction and to generate fragmented neoplastic tissue and cellular molecules having tumor specific antigens exposed thereon;

whereby said fragmented neoplastic tissue and cellular molecules in the presence of said immunoadjuvant form said cancer treatment product.

2. The cancer treatment product produced by the method of claim 1.

3. A product for encouraging an immune response in a tumor-bearing host comprising a mixture of fragmented neoplastic tissue and cellular molecules in the presence of an immunoadjuvant, said fragmented neoplastic tissue and cellular molecules having tumor specific antigens exposed thereon.

4. The product of claim 1 wherein said immunoadjuvant is selected from the group consisting of glycated chitosan, bacterial cell walls, liberated proteins, attenuated living bacteria, oils, modified chitosan, and non-toxic chitosan-like materials.

5. The product of claim 1 wherein said immunoadjuvant is glycated chitosan.

6. The product of claim 1 wherein said mixture is produced by inducing said neoplastic cellular destruction in the presence of said immunoadjuvant.

7. The product of claim 1 wherein said fragmented neoplastic tissue and cellular molecules are produced by photophysically destroying a neoplasm.

8. The product of claim 7 wherein said neoplasm is photophysically destroyed by a method comprising the steps of:

(a) introducing a chromophore into said neoplasm to obtain a conditioned neoplasm and

(b) lasing said conditioned neoplasm with a laser of a power and a wavelength and for a duration sufficient to activate said chromophore.

9. The product of claim 8 wherein said chromophore is selected from the group consisting of indocyanine green, DHE, m-THPP, $AIPcS_4$, ZnET2, and Bchla.

10. The product of claim 9 wherein said chromophore is indocyanine green.

11. The product of claim 8 wherein, in step (b), said conditioned neoplasm is lased in the presence of said immunoadjuvant.

12. The product of claim 8 wherein said method further comprises the step, prior to step (b) , of introducing said immunoadjuvant into said neoplasm.

13. A method of producing tumor specific antibodies, comprising the steps of:

(a) introducing an immunoadjuvant into a neoplastic tissue mass to obtain a conditioned neoplastic tissue mass;

(b) performing a physical therapy upon said conditioned neoplastic tissue mass to induce neoplastic cellular destruction and to generate fragmented neoplastic tissue and cellular molecules;

(c) allowing said vaccine to be dispersed systemically within a host to provoke the production of tumor specific antibodies; and

(d) collecting said tumor specific antibodies from host sera.

14. The tumor specific antibodies produced in accordance with the method of claim 13.

15. Tumor specific antibodies isolated from sera obtained from a tumor-bearing host, said tumor-bearing host having a neoplastic tissue mass, said neoplastic tissue mass having been conditioned with a chromophore and an immunoadjuvant and subsequently having been lased with a laser of a power and a wavelength and for a duration sufficient to activate said chromophore, thereby photophysically destroying said neoplastic tissue mass and exposing tumor specific antigens thereon to which said tumor specific antibodies combined to form an antigen/antibody complex.

16. A component or reagent for use in assays relating to cancer evaluation and treatment, comprising the tumor specific antibodies of claim 15.

17. An antigen/antibody complex formed by the combination of the tumor specific antibodies of claim 15 and antigens having an affinity therefor.

18. A method of collecting circulating free antigens for identification and synthesis to form the basis of a screening test for very early breast cancer, comprising contacting the tumor specific antibodies of claim 15 with an antigen containing source to allow for the formation of an antigen/antibody complex and subsequently separating and collecting said antigen.

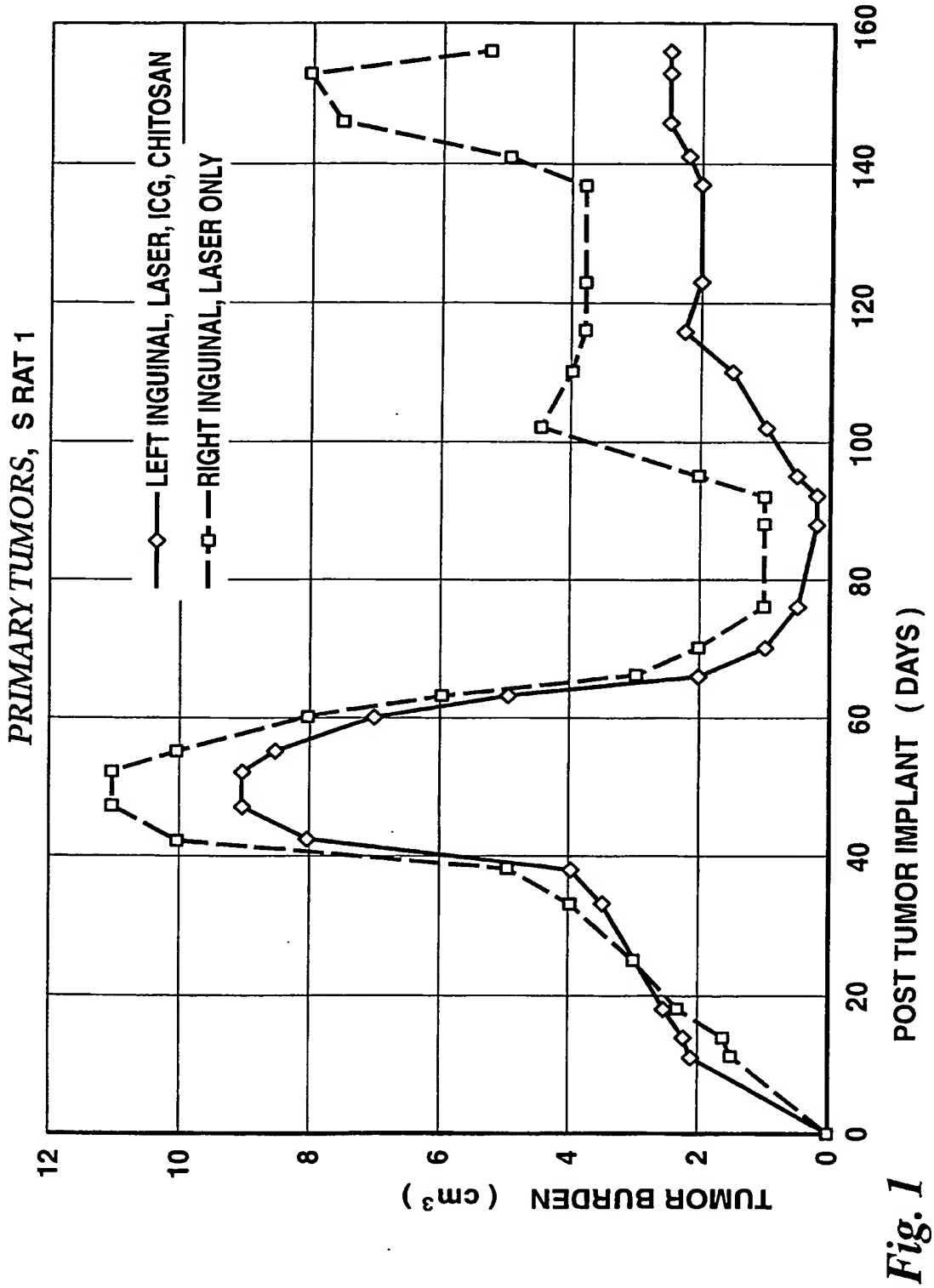
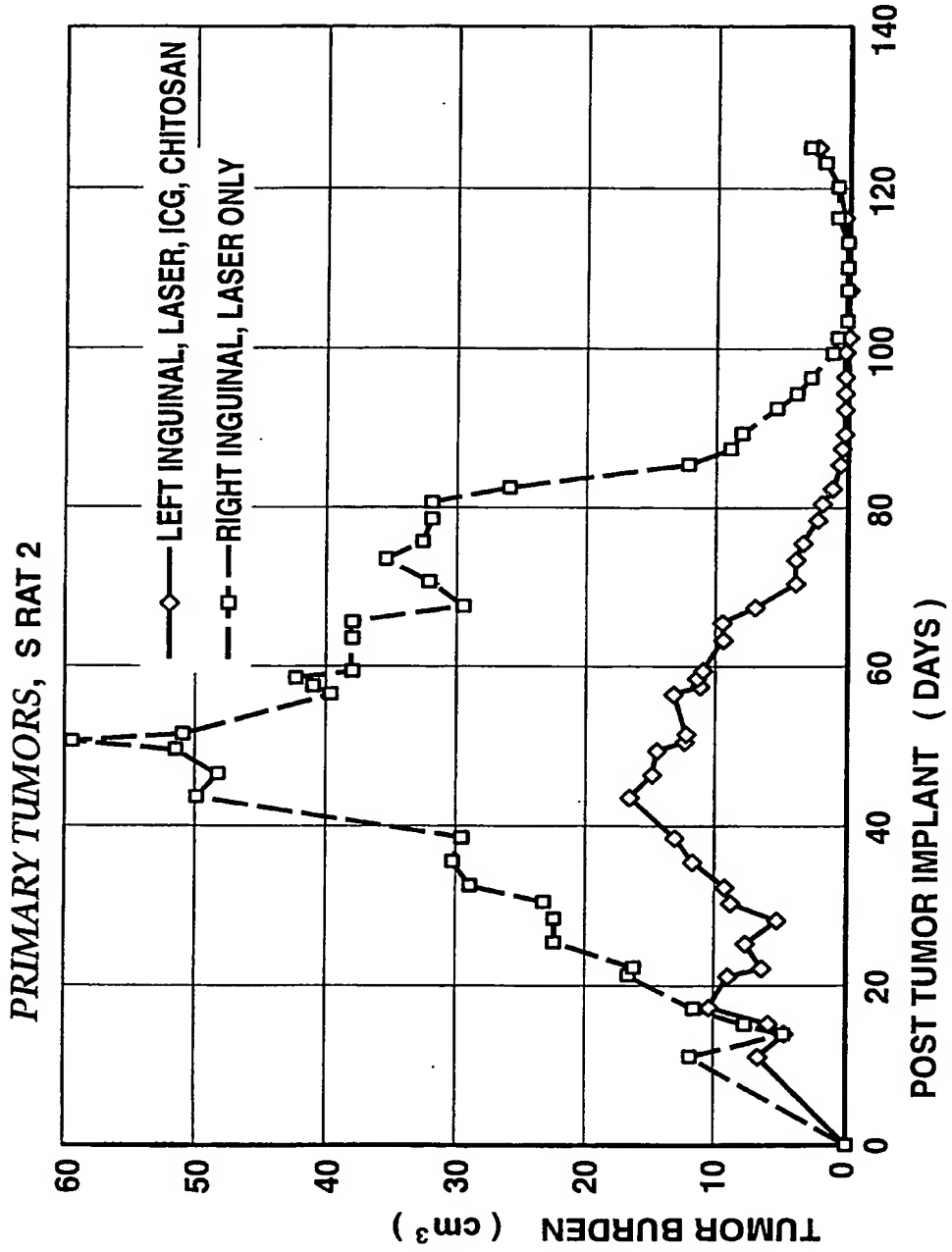

PRIMARY TUMORS, S RAT 1
LEFT INGUINAL, LASER, ICG, CHITOSAN
RIGHT INGUINAL, LASER ONLY
TUMOR BURDEN (cm³)
POST TUMOR IMPLANT (DAYS)
0
2
4
6
8
10
12
0
20
40
60
80
100
120
140
160

Fig. 1


PRIMARY TUMORS, S RAT 2
LEFT INGUINAL, LASER, ICG, CHITOSAN
RIGHT INGUINAL, LASER ONLY
TUMOR BURDEN (cm³)
POST TUMOR IMPLANT (DAYS)
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10
20
30
40
50
60
0
20
40
60
80
100
120
140

Fig. 2

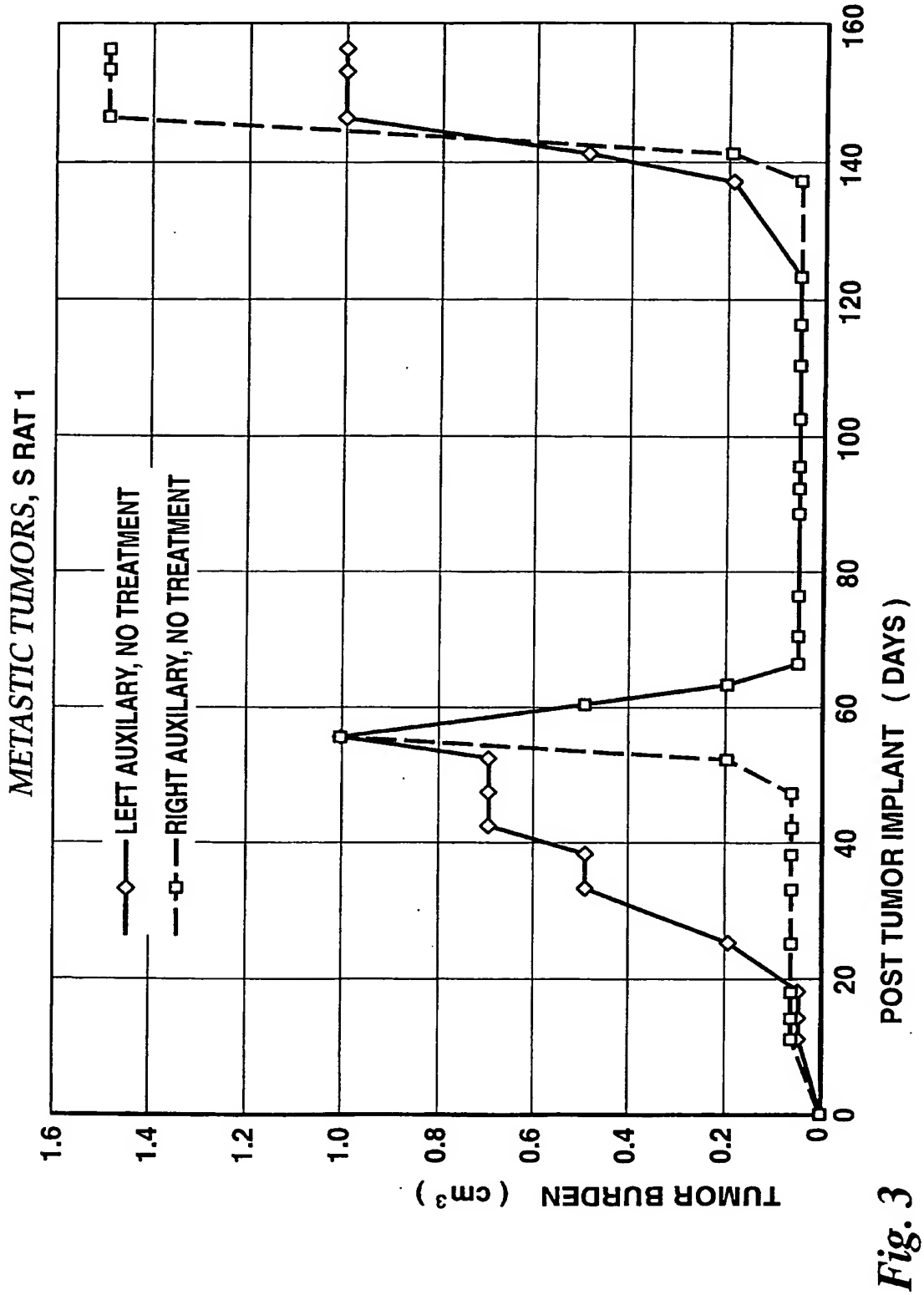
METASTIC TUMORS, S RAT 1
LEFT AUXILARY, NO TREATMENT
RIGHT AUXILARY, NO TREATMENT
TUMOR BURDEN (cm3)
POST TUMOR IMPLANT (DAYS)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
20
40
60
80
100
120
140
160

Fig. 3

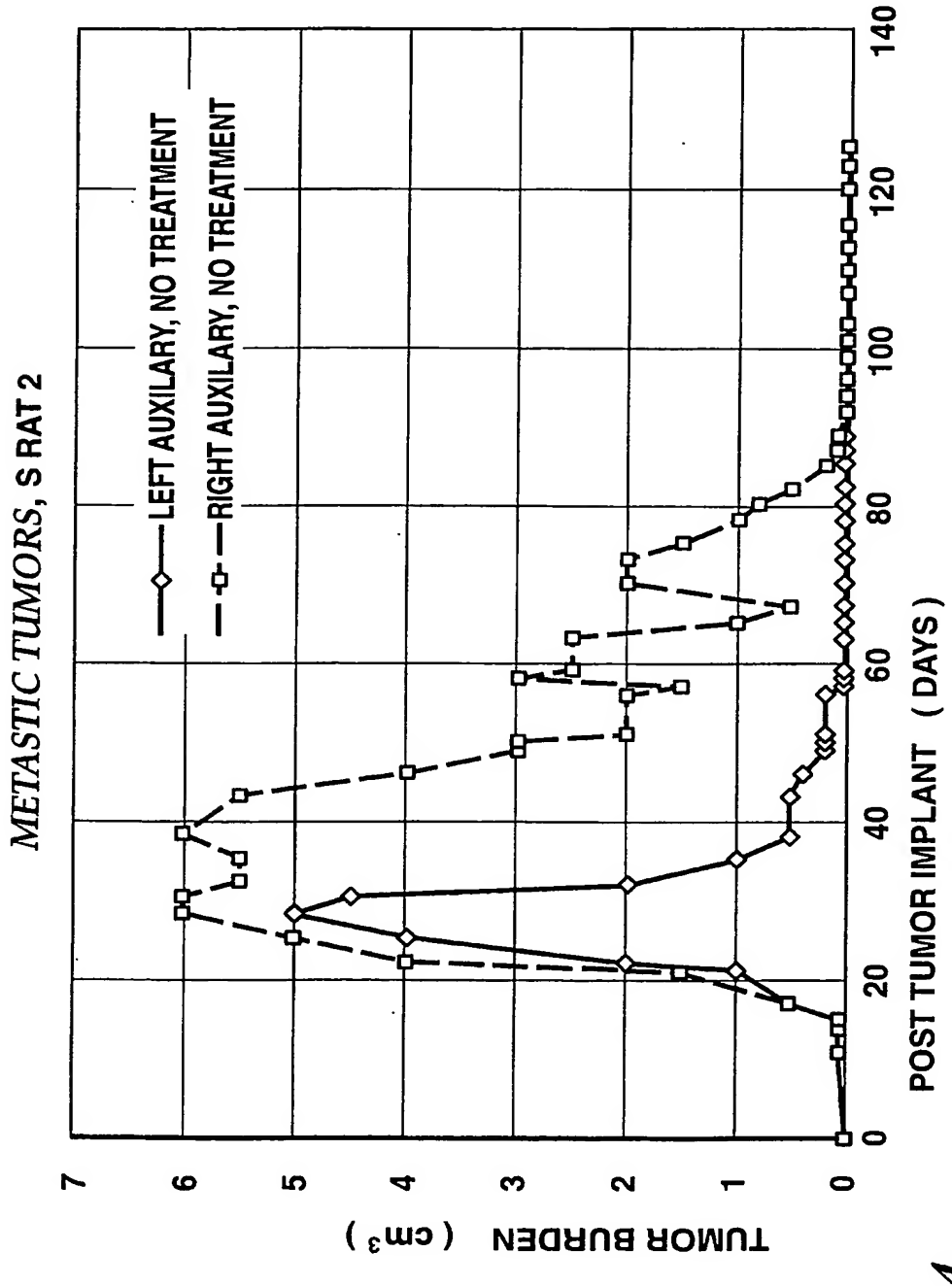
METASTIC TUMORS, S RAT 2
LEFT AUXILARY, NO TREATMENT
RIGHT AUXILARY, NO TREATMENT
TUMOR BURDEN (cm³)
0
1
2
3
4
5
6
7
POST TUMOR IMPLANT (DAYS)
0
20
40
60
80
100
120
140

Fig. 4

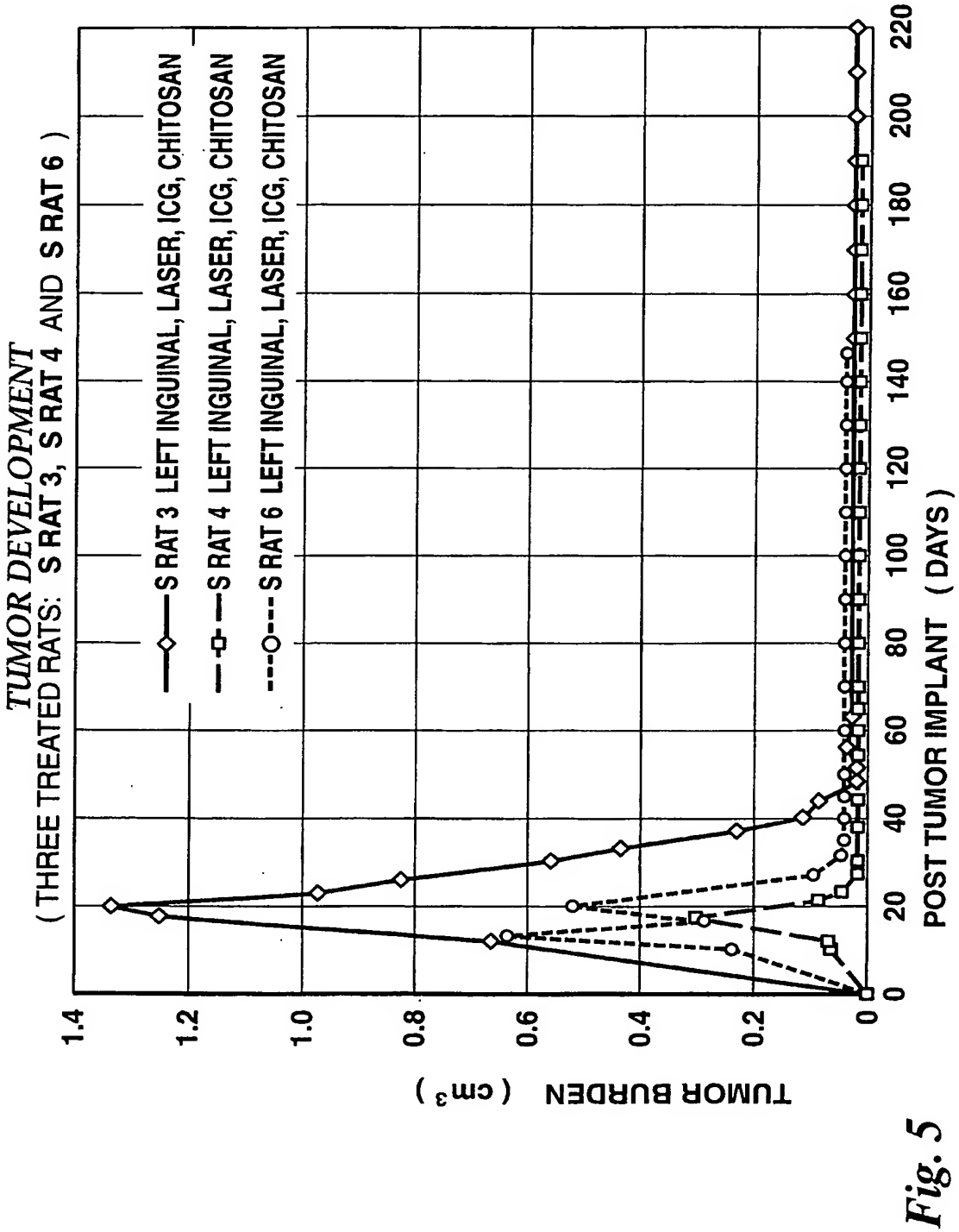
TUMOR DEVELOPMENT
(THREE TREATED RATS: S RAT 3, S RAT 4 AND S RAT 6)
S RAT 3 LEFT INGUINAL, LASER, ICG, CHITOSAN
S RAT 4 LEFT INGUINAL, LASER, ICG, CHITOSAN
S RAT 6 LEFT INGUINAL, LASER, ICG, CHITOSAN
TUMOR BURDEN (cm3)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
140
160
180
200
220
POST TUMOR IMPLANT (DAYS)

Fig. 5

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 98/09130

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/00 A61K41/00 C07K16/18 A61K39/395 G01N33/574

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 31237 A (WOUND HEALING OF OKLAHOMA) 10 October 1996 see the whole document ---	1-12
A	WO 96 17614 A (JON WANE CANCER INSTITUTE) 13 June 1996 see the whole document ---	1-18
A	CHEN W R ET AL: "Laser-photosensitizer assisted immunotherapy: a novel modality for cancer treatment." CANCER LETTERS, (1997 MAY 1) 115 (1) 25-30, XP002085724 see the whole document -----	1-12

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 November 1998	08/12/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/US 98/09130

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9631237 A	10-10-1996	AU 5256196 A	23-10-1996
		CA 2215978 A	10-10-1996
		CN 1186440 A	01-07-1998
		EP 0820305 A	28-01-1998
		US 5747475 A	05-05-1998
WO 9617614 A	13-06-1996	AU 4518196 A	26-06-1996